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Intracellular signalling crosstalk in the differentiation of F9 cells into extraembryonic endoderm

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Abstract

Mouse F9 cells differentiate into primitive extraembryonic endoderm (PrE) with retinoic acid (RA) treatment, as the result of an up-regulation of *Gata6*, which when translated directly activates *Wnt6*. Canonical Wnt signalling is required for PrE differentiation, and this, like most developmental processes, requires input from one or more additional pathways, including hedgehog (Hh). The Hh pathway is regulated by GATA6, and crosstalks positively/negatively with Wnt signalling. *Ihh* up-regulation in F9 cells accompanies PrE induction, but a role for GATA6 or Hh pathway activation in obligatory Wnt/ β -catenin signalling is not known. To address this, I show that RA induces *Ihh* and altered expression of Hh targets. Overexpressing *Gata6* alone significantly induced *Ihh*. When Hh signalling was blocked with cyclopamine, RA-induced differentiation, as analyzed by a decrease in TROMA-1 and DAB-2 signal, was significantly reduced, demonstrating requirement of the Hh pathway for PrE differentiation. Interestingly, each of SAG, BIO, and GLI3A overexpression failed to induce markers of PrE differentiation. These results indicate a regulatory role for Hh in PrE differentiation, as the pathway is required, but is not independently sufficient for this event.

Keywords: F9, Retinoic Acid, Hedgehog, IHH, GLI, WNT, Extraembryonic Endoderm, Primitive Endoderm, Differentiation, Crosstalk

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List of abbreviations

APC	Adenomatous polyposis coli
cAMP	Cyclic adenosine monophosphate
cDNA	Complimentary deoxyribonucleic acid
cGMP	Cyclic guanine monophosphate
CK1	Casein Kinase 1
Cyc	Cyclopamine
DAB-2	Disabled-2
db	Dibutyl
Dhh	Desert hedgehog
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSH	Dishevelled
ExE	Extraembryonic endoderm
FGF	Fibroblast growth factor
FL	Full length
FZD	Frizzled
GPCR	G protein coupled receptor
GSK3-β	Glycogen synthase kinase 3 β
GTP	Guanine triphosphate
Hh	Hedgehog
Hr	Hour
ICM	Inner cell mass
Ihh	Indian hedgehog
MAPK	Mitogen activated protein kinase
mM	Millimolar
mRNA	Messenger ribonucleic acid
nM	Nanomolar
PBS	Phosphate buffered saline
PCP	Planar cell polarity
PCR	Polymerase chain reaction
PE	Parietal endoderm
PKA	Protein kinase A
PrE	Primitive endoderm
PTCH	Patched
PTHrP	Parathyroid hormone related protein
q	Quantitative
RA	Retinoic acid
RAR	Retinoic acid receptor
RNA	Ribonucleic acid
RT	Reverse transcriptase
RXR	Retinoid X receptor
SAG	Smoothened agonist

SDS	Sodium dodecyl sulphate
Shh	Sonic hedgehog
SMO	Smoothened
TCF/LEF	T-cell specific transcription factor/Lymphoid enhance factor
TE	Trophectoderm
TROMA-1	Trophectodermal monoclonal antibody 1
VE	Visceral endoderm
WNT	Wingless/integrated
XEN	Extraembryonic endoderm
µg	Microgram
µM	Micromolar

CHAPTER 1

1 Introduction

1.1 *Mouse Early Embryogenesis*

Mammalian embryogenesis is an intricate process that requires various cellular signalling pathways. By the late blastocyst stage the embryo is comprised of the trophectoderm (TE) and inner cell mass (ICM). Trophectoderm cells mediate uterine implantation and give rise to the placenta, while the ICM gives rise to the epiblast and the primitive endoderm (PrE) [1]. The formation of the PrE is one of the first differentiation events in the embryo, giving rise to both parietal and visceral endoderm (PE and VE, respectively). During this event, VE forms adjacent to the epiblast and PE migrates down the TE [2]. Collectively, PrE, PE, and VE make up the eXtraembryonic ENdoderm (XEN) [1]. Cells within the ICM express both pluripotent and XEN genes *Nanog* and *Gata6*, respectively, in a salt and pepper-like pattern [3,4] eventually resulting in differentiation of PrE-lineage cells expressing *Gata6*, β -*catenin*, and *Dab-2* [5,1]. While not contributing directly to the organism itself, the VE and PE tissues form the visceral and parietal yolk sacs, respectively, essential to the survival of the embryo and for proper nutrient absorption. Yolk sacs, along with the placenta, also aid in protection of the developing embryo from mechanical damage [6]. The timing of these differentiation events, beginning prior to implantation, lends an air of difficulty to the *in vivo* study of the molecular signalling pathways controlling these events, and for this reason research investigating these tissues has primarily used *in vitro* cell models [7].

1.2 *F9 Teratocarcinoma cell model*

The F9 mouse teratocarcinoma cell line is derived from the collected stem cells of a teratoma, which was established through the injection of a day 6 mouse embryo into the testis of a 129/J mouse [8]. As cancer stem cells retain their capacity to differentiate into all three embryonic germ layers, F9 cells are unique, serving specifically as a model for the study of XEN development [8] [9,10]. Treatment of F9 cells with *all-trans*-Retinoic Acid (RA) differentiates cells to PrE [8], and this occurs because RA moves into the nucleus to bind to and activate retinoic acid receptors (RARs)/retinoid X receptor (RXRs), leading to the association of

coactivators and modified gene expression [7,8,11]. This modification in gene expression, particularly involving an increase in the transcription factors GATA6 and FOXA2 [1,9], effects changes in morphology and phenotype characteristic of PrE, making the F9 cell line a valuable model for these studies [8].

1.3 Hedgehog signalling and PrE

Three mammalian Hh ligand proteins exist in the mouse, Sonic (SHH), Desert (DHH), and Indian (IHH) [12], and each undergoes auto-proteolysis to form a cleaved (N-terminal), biologically active form, which acts as an extracellular signalling molecule and morphogen [13]. Hh, when bound to the 12-pass transmembrane receptor Patched (PTCH), relieves inhibition of a seven-pass transmembrane GPCR, Smoothened (SMO). This SMO activation results in an upregulation and/or posttranslational processing of GLI transcription factors; a repressive form (i.e., GLI3R) to an active form (i.e., GLI3A) involving a poorly understood interplay between Protein Kinase A (PKA), Glycogen Synthase Kinase 3- β (GSK3- β), and Casein Kinase (CK1) [14,15]. GLIs then move into the nucleus to upregulate Hh-specific target genes (Fig. 1), some of which include *c-Myc*, *Cyclin D1* (*Ccnd1*), *Ptch*, and other *GLIs* [16].

Hedgehog (Hh) signalling is involved in VE differentiation [17], but its role in PrE formation is largely uninvestigated. It has been established that primary cilia, small projections from the cellular membrane housing signal transduction of the hedgehog pathway, exist in the epiblast of the mouse embryo following implantation, but extraembryonic endoderm lineages, including VE, remain unciliated [18,19]. F9 cells specifically, when undifferentiated, are positive for the primary cilia marker gamma tubulin [20], but whether the *in vivo* pattern recapitulated by XEN-derived stem cells is shared by PrE-differentiated F9 cells remains unknown [18]. This *in vivo/in vitro* lack of primary cilia, the historically-considered site of Hh signalling, in ExE lineages raises yet-unanswered questions as to the timing and mechanism by which this pathway is regulated in ExE lineages. *In vitro* studies suggest that Hh signalling in the mouse embryo patterns ExE, and these studies reported that when F9 cells are treated with RA, the expression of *Ihh* increases as the cells differentiate into a PrE state [21]. *In silico* analysis from our lab revealed that the *Ihh* promoter has a putative binding site for GATA6 [22], a transcription factor required for the differentiation of embryonic and extraembryonic endoderm

that is directly involved in up-regulating *Wnt6* [1,9]. These preliminary results implicate Hh signalling in PrE differentiation through the Ihh ligand.

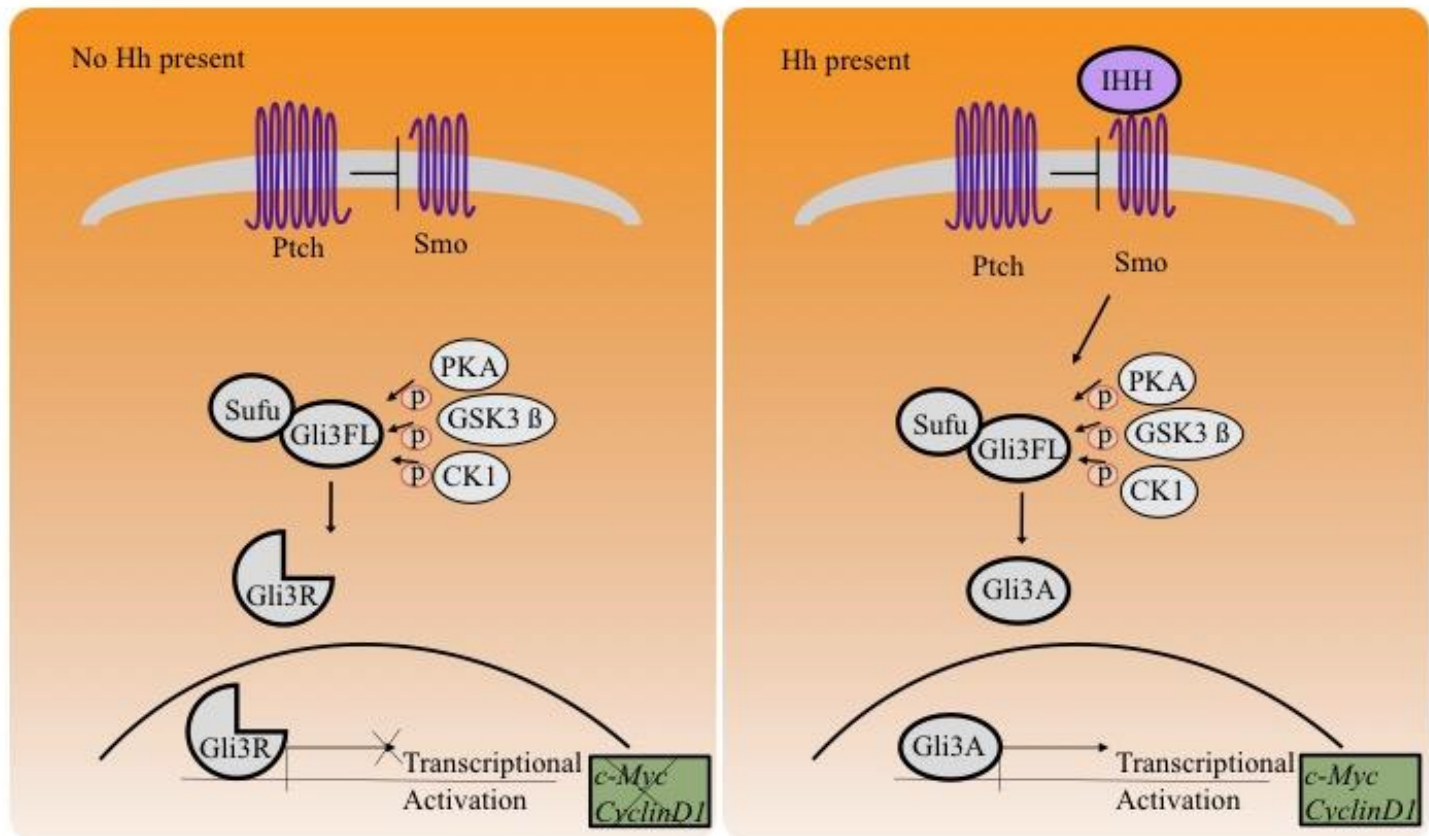


Figure 1. **Hedgehog signalling pathway.** When a Hh ligand is absent, the PTCH receptor inhibits the Smoothed receptor. In this case, GLI-full length (FL) protein is held in the cytosol by SUFU, where it is primed and phosphorylated by PKA at specific serine-threonine sites. Priming is followed by phosphorylation by GSK3- β and CK1, resulting in proteolytic degradation of the C-terminal end of the GLI protein, and a repressive transcription factor that can then move to the nucleus. When Hh is present and bound to PTCH, Smoothed is uninhibited through unknown mechanisms, and the priming and phosphorylation of GLI by PKA is altered, resulting in a new phosphorylation signature on GLI as well as a new GSK3- β and CK1 phosphorylation status. In this case, GLI is not degraded in part by the proteasome, and is free to move into the nucleus to act as a transcriptional activator. Based on [23].

1.4 *GLI protein post-translational modification*

Smo activation leads to post-translational modification of Gli transcription factors, of which there are three in vertebrates; GLI1, GLI2, and GLI3. While all three function as mediators of downstream Hh signalling, these proteins can act as activator only (GLI1; expression target of active Hh signalling), preferential activator (GLI2; context dependent), or preferential repressor (GLI3; during inactive Hh signalling) [14]. These three transcription factors differ in that GLI1 structure includes solely a transactivation domain, while GLI2 and GLI3 protein structures include both a transactivation domain as well as a repressor domain; in the event of active Hh signalling, GLI2/3 are phosphorylated to an active form, while in the absence of Hh signalling, the transactivation domain is proteolytically degraded, leaving only a functional repressor domain [24,25]. My focus in this study is GLI3, due to recent literature describing this protein as both a regulator of pluripotency/differentiation and the Wnt pathway during differentiation in the mouse [26,27].

The activating or repressive status of GLI3 depends on priming and regulatory phosphorylation patterns determined by kinases CK1, GSK3- β , PKA [14]. Specifically, GLI3 is phosphorylated at 6 specific conserved sites by PKA in the absence of active hedgehog signalling, forming the repressive transcription factor [24]. During active signalling, however, all 6 of these PKA-phosphorylated sites are de-phosphorylated, while a separate set of residues/sites are phosphorylated, leading to the formation of the active transcription factor [24]. Thus, Niewiadomski et al. (2014) dubbed the regulation of GLI transcription factors as “phospho-coded” and therefore subject to a graded control of active:repressive activity, consistent with the potential morphogen-like pattern of Hh signalling.

1.5 *Wnt signalling and PrE*

Wnt signalling plays many different roles during development, ranging from differentiation to organogenesis [28], and this signalling can take place via three different transduction pathways; canonical/ β -catenin signalling, as well as two different non-canonical transduction pathways; Planar Cell Polarity (PCP) and the Wnt/ Ca^{2+} signalling pathways [28].

The diversity of signalling outcomes by this pathway is made clear by the existence of 19 mammalian WNT ligands [29] and 10 mammalian Frizzled (FZD) receptors [30]. Regardless of varied downstream signal amplification across these three Wnt signalling pathways, Dishevelled (Dsh) remains a constant player following ligand-receptor interaction [28], while the non-canonical pathways, importantly, can signal through active Fzd receptors without leading to regulation of β -catenin.

Non-canonical WNT signalling can alter cell polarity leading to altered cell arrangement, physiological function and morphology, in the case of the PCP pathway. Of interest, is the role this pathway plays in gastrulation through activation of the GTPases Rac and Rho [28,31] leading to alterations in the cytoskeleton and eventually cell migration. The non-canonical Wnt/ Ca^{2+} pathway similarly contributes to gastrulation via G-protein signalling through G-protein Coupled Receptor (GPCR)-functioning FZD receptors [28,32] leading to TGF- β activation as well as inhibition of canonical Wnt signalling [33].

Canonical WNT signalling involves binding of a WNT ligand to a FZD receptor with its co-receptor, LRP. This interaction promotes the inactivation of a destruction complex consisting of Adenomatous Polyposis Coli, GSK3- β , and CK1, and β -catenin, allowing the latter to accumulate and eventually translocate to the nucleus, where it serves as a co-activator of transcription with TCF-LEF (Fig. 2) [34]. Our lab has shown that GATA6 activates canonical Wnt- β -catenin signalling, upregulating the Wnt-target Disabled-2 (DAB-2), and differentiating F9 cells to PrE. We have also discovered that inhibition of Hedgehog signalling results in an attenuation of WNT signalling at the level of TCF/LEF activity (Deol, Cuthbert, et al.; *submitted*), demonstrating cross-talk between the two signalling pathways during PrE differentiation.

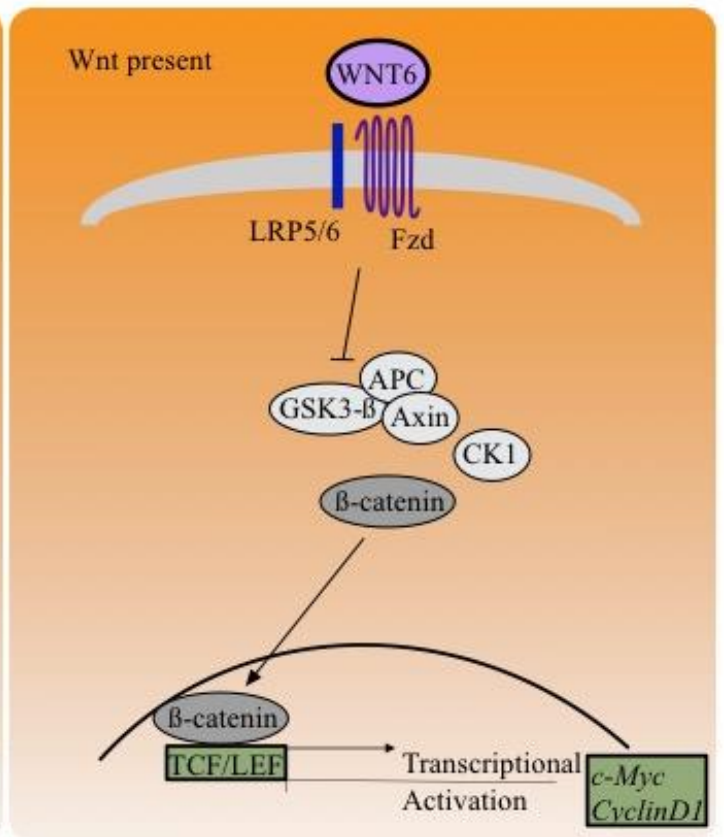
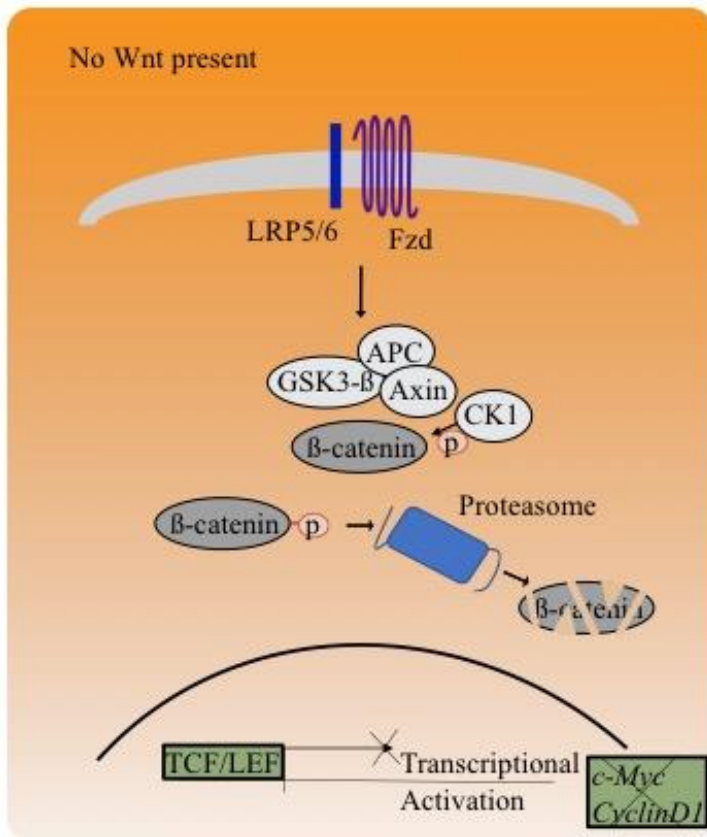


Figure 2. **Canonical Wnt signalling pathway.** When a Wnt ligand is absent, the FZD receptor is inactive, and in this case β -catenin is held in the cytosol by a destruction complex consisting of GSK3- β , APC, AXIN, and CK1. The destruction complex is responsible for phosphorylating β -catenin, resulting in its ubiquitination and destruction by the proteasome. In this event, β -catenin is unable to move to the nucleus to act as a TCF/LEF transcriptional co-activator. When a Wnt ligand is present and interacting with a FZD receptor, the destruction complex is inhibited through Dishevelled (not shown in image), leading to the release of β -catenin, and allowing it to move to the nucleus to act as a transcriptional co-activator for target genes. Based on Apocell.com, 2016.

1.6 *FGF/MAPK signalling and PrE*

FGF/MAPK signalling involves binding of Fibroblast Growth Factor (FGF) to a receptor tyrosine kinase (FGFR), resulting in downstream phosphorylation events, activating ERK1/2, and eventual activation of transcription factors [4]. FGF/MAPK signalling is required in the developing mouse embryo by E4.0-4.5 [4] and without it, GATA6-expressing PrE fails to form. Verheijen et al. [6] reported that PKA, an upstream inhibitor of MAPK signalling at the level of RAS, must be inactivated to allow PrE formation. Furthermore, PKA is activated by parathyroid hormone related protein (PTHrP) and cAMP for subsequent formation of PE [4]. These results indicate there is an absence of PKA signalling during PrE formation and that this is relieved during the switch to PE, which is supported by the fact that there is a decrease in cAMP levels in PrE as compared to undifferentiated and PE-differentiated F9 cells [35].

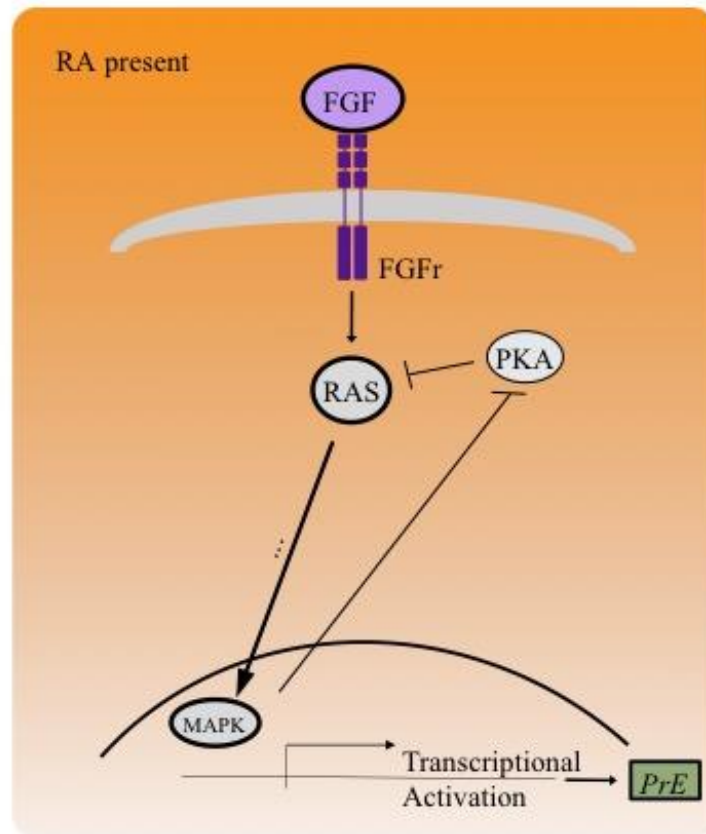


Figure 3. **FGF/MAPK signalling and PrE differentiation.** When RA is present, the FGF ligand is bound and activates the FGF receptor, a receptor tyrosine kinase. In this event, a series of kinases are sequentially activated, leading to activation of MAPK (ERK1/2), which phosphorylates and regulates target substrates contributing to cell differentiation and proliferation. Of note, is the ability of active PKA to inhibit this pathway at the level of RAS, resulting in inhibited activation and PrE differentiation. Alternatively, active MAPK inhibits PKA, maintaining the potential for PrE differentiation until external signalling results in PKA activation by cAMP, and thus PE differentiation.

1.7 *Hedgehog, Wnt, and MAPK signalling crosstalk*

Activation of both Hedgehog and Wnt signalling pathways is dependent on 7-pass transmembrane proteins, either Smo or Fzd, respectively, which belong to the frizzled family of receptors [30]. It is important to note that while not all members of the frizzled family act as such, both SMO and FZD meet the criteria to function as a GPCR, i.e., having an external N-terminus and internal C-terminus inclusive of motifs capable of phosphorylation and coupling to G-proteins [36]. Of interest is the fact that the SMO receptor is not ligand-responsive. This receptor instead is activated through relief of inhibition by the Ptch receptor through mechanisms that are poorly understood [16]. For this reason, while the mechanism by which SMO activation leads to alterations in the Hh pathway also remains elusive, research suggests that SMO may act as a G-protein coupled receptor (GPCR), and as a possible regulator of cAMP and PKA activity [37,38] in mammalian cells. Alternatively, the FZD receptor can act non-canonically to alter intracellular Ca^{2+} levels as well as cGMP via interaction with $\text{G}\alpha$ proteins in the zebrafish embryo [36]. Similar outcomes were obtained using a receptor chimera F9 cell line, demonstrating that this role for the Fzd family of receptors as functional GPCRs also occurs in mammalian cell lines [32]. MAPK is activated in the absence of PKA and cAMP, which as previously stated, is required for F9 cells to differentiate to PrE [4,6]. This common predicted dependence on cyclic nucleotide signalling underlines the individual requirement for each of the Hh, Wnt, and MAPK pathways in PrE differentiation.

Similarity exists between the Hh and Wnt pathways, including ligand post-translational regulation, to receptor similarity, and downstream signalling amplification via phospho-modified transcriptional regulator(s). As such, it is not surprising that crosstalk between these downstream signal amplifiers is observed; for example, in mouse embryonic stem cells, GLI3 physically interacts with NANOG, resulting in a balancing effect between pluripotent and differentiated states, with this balance being tipped when Hh signalling becomes active resulting in differentiation [26]. Similarly, a physical interaction between GLI3 and β -catenin occurs in the differentiating neural tube, whereby GLI3R physically interacts with and reserves β -catenin from functioning as a transcriptional activator, even in the presence of Wnt [27]. These examples of crosstalk between the Hh and Wnt pathways contributing to differentiation have provided strong impetus for the studies outlined in this thesis.

1.8 Objectives of study

The objectives of this study were to determine: 1) if *Ihh* is the Hh ligand to respond to the RA signal in the F9 cell line, and determine the response of Hh signal-responsive genes in the F9 cells with RA treatment. 2) to determine whether the GATA6 master regulator is sufficient/capable of inducing expression of the *Ihh* ligand. 3) to determine whether Hh signalling is sufficient and/or necessary for differentiation of F9 cells to PrE, and if this was accompanied by a change in the GLI3A:GLI3R ratio.

Given what is known about the Hh signalling pathway and differentiation of PrE,

I hypothesize that Ihh responds to RA signalling in F9 cell PrE differentiation, and that activation of the pathway serves to post-translationally modify GLI3. I further hypothesize that this post-translational modification to GLI3 is regulated by kinases GSK3- β and PKA through a common dependence on cyclic nucleotide signalling regulation as a source of crosstalk between the Hh and Wnt pathways.

I have discovered that the components of the Hh signalling pathway are expressed and several are altered in the F9 teratocarcinoma cell line in response to RA during PrE differentiation, specifically the response of *Ptch* receptor, and GLI3 transcription factor. I have also demonstrated that *Ihh* is present in the mouse blastocyst at both the transcript and protein level at the appropriate time-point in development, at E3.5. After determination that GATA6 is sufficient for upregulation of *Ihh*, it was determined that Hh signalling is necessary, but not sufficient for differentiation of F9 cells to PrE. Furthermore, I observed that GLI3 protein appears to undergo post-translational modifications during the 96hr timeline following RA treatment, leading to PrE. Unfortunately, while GLI3A overexpression appeared to result in changes in cytokeratin-8 and 18 intermediate filament levels, it was not sufficient alone to fully differentiate cells to PrE, and for this reason the regulation of the pathway at the level of GSK3- β was investigated. Inhibition of GSK3- β as a means of Hh pathway activation yielded no PrE differentiation. Preliminary investigation of a target higher in the pathway, at the level of the GCPR, demonstrated that mRNAs encoding inhibitory protein *Gai1* and *Gai2* (*Gnai1* and *Gnai2*, respectively) were expressed and *Gnai1* was altered with treatment of RA in the F9 cell

line as well as the E3.5 mouse blastocyst. Together, the objectives of this study sought to solidify a role for Hh signalling in PrE differentiation.

CHAPTER 2

2 Materials and Methods

2.1 *Cell culture, mouse blastocyst, transfection, and treatment*

Mouse F9 teratocarcinoma cells (ATCC) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Lonza) enriched with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin antibiotic (PS; Lonza). Cells were seeded in tissue culture (TC) treated 60mm plates (BD Falcon) for subsequent protein isolation. When cells were approximately 40% confluent they were treated with either 10^{-7} M retinoic acid (RA; Sigma) overnight, and then subsequently with 5 or 10μ M Cyclopamine (Cyc; EMD Millipore), 5 or 10 nM Smoothened Agonist (SAG; EMD Millipore), 10nM 6-Bromoindirubin-3'-oxime (BIO; Sigma-Aldrich) or treated simultaneously (immunoblot and RT-qPCR). Control and treated cells were incubated at 37°C and 5% CO₂ for 4 days.

Cells were transfected with pcDNA3.1 empty vector with either 10^{-7} M DMSO or RA, 10^{-7} M DMSO and pcDNA3.1-*Gata6* (Morrisey Lab), or 10^{-7} M DMSO and pcDNA3.1-*DN2Gli3* (GLI3A; Briscoe Lab) constructs using Lipofectamine2000 according to the manufacturer's recommendations (ThermoFisher Scientific). Briefly, 10 μ L of Lipofectamine2000 was mixed with a total of 4 μ g of expression constructs to transfect cells grown to 60% confluence in 35 mm tissue culture treated plates (BD Falcon). Transfected cells were then passaged 24hr later to 60 mm TC dishes. Cells were treated with 0.05% Dimethyl sulfoxide (DMSO; vehicle control), or 10^{-7} M RA. All cells were incubated at 37°C and 5% CO₂. All experiments are representative of triple biological replicates, as defined by cells of different passage number.

2.2 *Qualitative and quantitative reverse-transcription PCR*

To determine relative steady-state mRNA levels in cells, total RNA was isolated from F9 cells at 24, 48, 72, and 96 hours post-treatment/transfection using the RNeasy Mini Kit (Qiagen). RNA was reverse transcribed into first strand cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Oligodeoxynucleotide primers were designed to amplify

the mouse *Shh*, *Dhh*, *Ihh*, *Wnt6*, *Dab2*, *Gata6*, *Smoothed*, *Patched1*, *Patched2*, *Sufu*, *Gli1*, *Gli2*, and *Gli3* nucleotide sequences (Appendix 1; Ct values in Appendix 2). Primers to the constitutively expressed ribosomal gene *L14* were used as controls. qRT-PCR was conducted with the cDNAs as templates under the following reaction conditions: 500 nM of each reverse and forward primer, SensiFAST SYBR Mix (FroggaBio), and 1 μ L of appropriate cDNA. Samples were analyzed with the CFX Connect Real-Time PCR Detection System (Bio-Rad) using the comparative cycle threshold (Δ/Δ Ct) method, and relative expression values were obtained from steady-state mRNA levels normalized to *L14* mRNA. Relative expression values were subsequently normalized to DMSO/control plasmid treatment(s) to determine fold change. RT-PCR amplified cDNA samples were for 40 cycles, and run on 2% agarose gels containing RedSafe (FroggaBio) nucleic acid staining solution and visualized using the BioRad ChemiDoc Touch Imaging System. Primers for constitutively expressed L14 were used again as controls, with no template controls remaining absent of any bands as a further control. All experiments were completed in triple biological replicates, as defined by different passage number as well as triple technical replicates.

2.3 Immunoblot analysis

Cells were lysed in 300 μ L of 2% sodium dodecyl sulfate (SDS) buffer containing 62.5mM Tris-HCL pH 6.8, 10% glycerol, 5% Mercapto-2-ethanol, and 1X Halt Protease Inhibitor Cocktail (Thermo Scientific). Protein concentrations of lysates were measured using a Bradford assay (Bio-Rad) and equivalent amounts were separated on denaturing 6% or 10% polyacrylamide gels and transferred to nitrocellulose membranes (Biotrace; Pall Corp.). Membranes were blocked in 5% skim milk and then probed with primary antibodies directed against TROMA-1 (1:50; Developmental Studies Hybridoma Bank), DAB-2 (1:10,000; Santa Cruz), OCT-4 (1:10,000; Cell Signalling), IHH (1:10,000; Abcam), GLI3 (1:10,000; R&D Systems). and β -actin (1:10,000; Pierce). After extensive washes, membranes were probed with the appropriate secondary antibodies, washed and then signals detected using the Luminata Classico Western HRP Substrate (Millipore). All experiments were completed in triple biological replicates, as defined by different passage number, each with DMSO as negative control, and probed with β -actin as loading control.

2.4 Microscopy

Cells were fixed on glass-bottom 12-well plates (MatTek) with 4% paraformaldehyde (Electron Microscopy Sciences), permeablized with 0.1% Triton X-100, and finally blocked in 1% bovine serum albumin. Cells were incubated overnight at 4°C with TROMA-1 antibody (1:50) and Alexa488-conjugated anti-rat antibody (ThermoScientific) for 2 hours, followed by DAPI (Molecular Probes) and Vectashield antifade reagent. All reagents were diluted in phosphate buffered saline. Cells were examined a using Zeiss LSM 5 Duo Vario Microscope with both ZEN and Image-Pro Premier 3D software. DMSO and RA served as negative and positive controls, respectively.

2.5 Statistical Analysis

Data from all experiments are representative of at least three independent biological replicates, as defined by differing passage number, performed on separate occasions. Embryo experiments were the result of three separate E3.5 mouse embryo pools consisting of 20-35 embryos each. Data comparisons between the control and treated groups were performed using a one-way ANOVA with Tukey's honest significant difference (HSD) post-hoc test (SPSS Statistics for Windows Version 19.0, IBM Corp. Released 2010, Armonk, NY). Significance is denoted by letters, numbers, symbols, or a combination, as indicated. P-values were considered statistically significant at the 0.05 level and statistical data are presented as the mean \pm S.E. PCR amplification products were confirmed via Next-Generation Sequencing at the London Regional Genomics Centre.

CHAPTER 3

3 Results

3.1 Hedgehog pathway component expression in F9 cells

F9 cells treated with RA differentiate into PrE, and this is accompanied by an increase in the expression of *Ihh* [21]. Two other Hh genes are expressed in mouse and for this reason the expression profiles of *Ihh*, *Shh*, and *Dhh* were examined in F9 cells. Total RNA was collected from cells treated with dimethyl sulfoxide (DMSO) (vehicle control) or RA from 24 to 96 hours, post-treatment. Results show there was a significant increase in *Ihh* expression, relative to DMSO controls, following RA treatment at 24 hours ($p = 0.03$; Fig. 1A). In contrast, results for *Shh* and *Dhh* with relatively high Ct values (36 and 33, respectively) as compared to *Ihh*, indicate negligible changes in the levels of mRNAs encoding these genes following RA treatment (Appendix 2). Thus, *Ihh* was the only RA-responsive Hh family member and was thus selected as the proposed candidate involved in the differentiation of F9 cells to PrE, and increased *Gata6*, *Wnt6*, and *Dab-2* expression (Fig. 1B-D). Having observed that *Ihh* was induced by RA, I next assessed effects to the expression of components of the Hh pathway including *Smo*, *Ptch1*, *Ptch2*, *Sufu*, *Gli1*, *Gli2* and *Gli3*. No significant changes were detected in either *Smo* or *Ptch1* expression (Fig. 1E). As expected under conditions of active Hh signalling [25], *Ptch2* expression increased significantly ($p=0.034$) in cells treated with RA for 24-48 hours, before dropping to levels of undifferentiated cells (Fig. 1E). This pattern of *Ptch2* expression confirms recent literature demonstrating an independent role of *Ptch2* over *Ptch1* during early development in the chick embryo [39]. *Gli1* was included in this analysis as its expression was expected to increase because of activated Hh signalling. No statistically significant increase was observed between the DMSO control and RA-treated cells at 24-96 hours, and surprisingly expression levels dropped after 24-hours (Fig. 1F). *Gli2* and *Gli3* were also examined and while no biologically significant changes (fold-changes < 3) were detected between RA and DMSO treatments for *Gli2*, *Gli3* mRNA expression increased between 24-72 hours ($p=0.008$; Fig. 1F). Increased expression of *Gata6*, the master regulator of endoderm and extraembryonic endoderm formation, its target *Wnt6* and Wnt target *Dab2* (Hwang and Kelly, 2012; Supplementary Fig.

1B-D) prompted the inclusion of *c-Myc* and *Ccnd1* into the overall analysis, since both are WNT and Hh signalling target genes [40–44]. The expression of *c-Myc* and *Ccnd1* increased by 24 hours post-RA treatment, but these levels fell sharply by 48 hours (Fig. 1G). Although RA treatment up-regulated the expression of *Ihh*, as well as Hh signalling readouts, *Ptch2*, *c-Myc*, and *Ccnd1*, it was necessary to confirm that *Ihh* is expressed early in the mouse blastocyst, before subsequently investigating how *Ihh* might be regulated in embryogenesis.

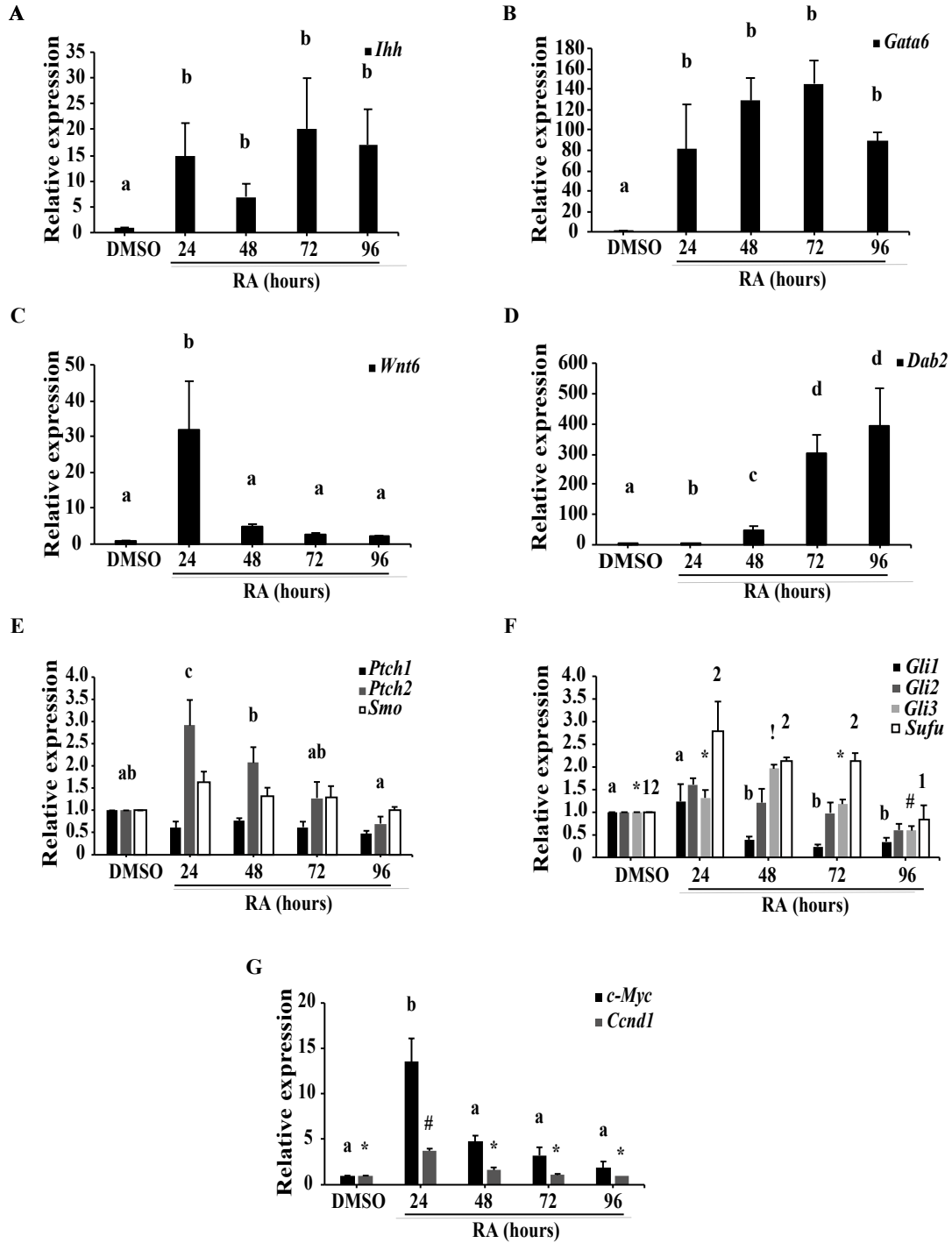


Figure 4. **Hedgehog pathway component expression during RA-induced F9 cell differentiation.** Hh pathway component and target gene mRNA levels fold-change in response to retinoic acid-induced PrE differentiation. Total RNA was extracted from F9 cells treated with retinoic acid (RA) to induce primitive endoderm at 24, 48, 72, and 96 hrs post-treatment, and relative mRNA levels of: (A) Indian (*Ihh*) hedgehog (B) *Gata6*, (C) *Wnt6*, (D) *Disabled-2* (*Dab2*), (E) *Patched-1* (*Ptch1*), *Patched-2* (*Ptch2*), and *Smoothened* (*Smo*), (F) *Gli1*, *Gli2*, *Gli3*, and *Sufu* (G) *c-Myc* and *Ccnd1* were examined by qRT-PCR. Data are representative of three independent experiments \pm SEM. Letters, asterisks, and #'s indicate significant difference ($p < 0.05$) above and below the DMSO control as tested by One-Way ANOVA followed by a Tukey test relative to the constitutively expressed *L14* gene.

3.2 Presence of *Ihh* in E3.5 blastocyst

A role for *Ihh* in early mouse embryogenesis was established by Becker et al. [21], and later by Grabel et al., [17]. These studies reported the presence of *Ihh* in the E6.5 mouse blastocyst, and later a role for the ligand in VE differentiation. *Ihh* expression was also reported in the F9 cell model [17], where the authors showed an increase in both protein and mRNA levels following RA treatment. For my studies cDNA and protein lysates from pools of 20-35 mouse E3.5 blastocysts were kindly donated by the Betts Lab (Department of Physiology and Pharmacology, UWO). Blastocyst cDNAs were first investigated with RT-PCR using primers to *Ihh*. A 150 bp amplicon was detected and when sequenced at the London Regional Genomics Centre, it was determined that the amplified product was *Ihh* (Fig. 2B). To corroborate these results, I conducted western blots using antisera against IHH and β -ACTIN, and detected IHH protein levels at E3.5 (Fig. 2A). Thus, both protein and mRNA analyses demonstrate *Ihh* presence at E3.5, which is earlier in development than previously reported [21].

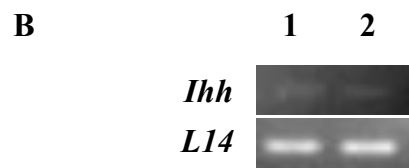
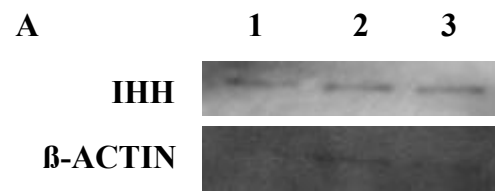


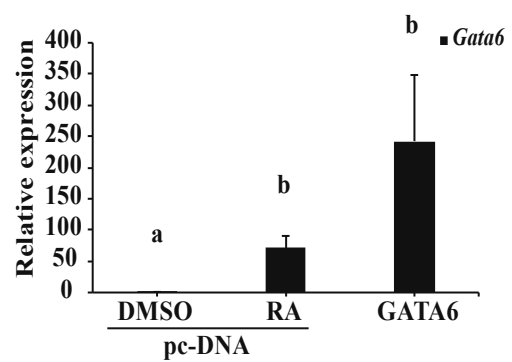
Figure 5. **Presence of *Ihh* in E3.5 blastocyst.** Presence of (A) *Ihh* mRNA and (B) IHH protein in E3.5 mouse blastocysts was assayed using RT-PCR and immunoblot, respectively. Data are representative of three independent experiments, and each cDNA and protein lysate sample consisted of 25-35 pooled embryos.

3.3 GATA6 regulates *Ihh* during RA-induced F9 cell differentiation

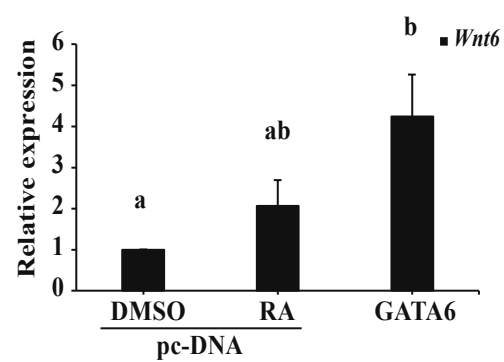
Our lab has determined that the *Ihh* promoter contains putative binding sites for GATA6 (unpublished). GATA6 binds directly to the *Wnt6* promoter to regulate its expression [9]. In combination with a burst of ROS [45] which is likely produced by NADPH oxidases and is necessary for active Wnt signalling [46,47] to allow F9 cells differentiate into PrE. Given this hierarchy and the presence of a putative GATA6 binding site in the *Ihh* promoter, I hypothesized that GATA6 is responsible for the increase in *Ihh* seen coincident with differentiation.

To address this question, F9 cells were transfected with pcDNA3.1 (empty vector control) or pcDNA3.1-*Gata6*, or transfected with pcDNA3.1 and treated with RA. Total RNA was collected 96 hours post-treatment and reverse transcribed into cDNA for qRT-PCR analysis with *L14*, *Gata6*, *Ihh*, *Wnt6*, and *Dab2* primers. As expected, *Gata6* expression in RA-treated cells was significantly greater than the DMSO controls ($p=0.0075$), and there was no significant difference in the levels between RA and those due to *Gata6* overexpression ($p=0.206$; Fig. 3A). RA and *Gata6* overexpression each independently induced *Wnt6* and *Dab2*, with p values of 0.036 for *Wnt6* (4.2-fold) and 0.006 (359-fold) for *Dab2* with *Gata6* overexpression as compared to empty vector (Fig. 3B and 3C, respectively), indicative of PrE differentiation. *Gata6* also induced *Ihh* expression to significantly higher levels than the DMSO controls ($p=0.040$), and this was not significantly different to those induced by RA ($p=0.911$; Fig. 3D).

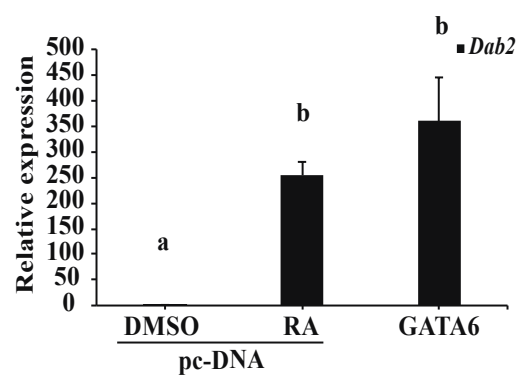
A



B



C



D

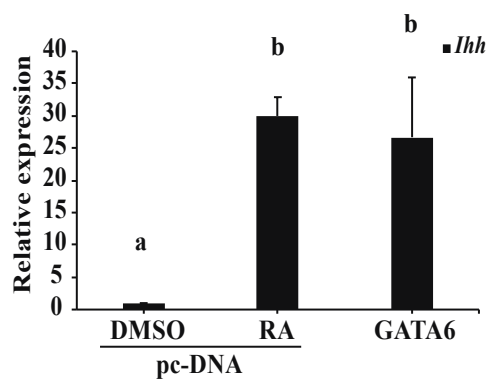


Figure 6. GATA6 positively regulates *Ihh* during RA-induced F9 cell differentiation. F9 cells were transfected with a pcDNA3.1 empty vector (control) or pcDNA3.1-*Gata6*. Total RNA was collected 96hr later and cDNA analyzed by PCR using primers designed to amplify (A) *Gata6*, (B) *Wnt6*, (C) *Dab2*, and (D) *Ihh*. The data presented are fold-change as compared to empty vector, FMSO control, from three independent experiments \pm SEM. Letters indicate significant difference ($p < 0.05$) as tested by One-Way ANOVA followed by a Tukey test relative to *L14* and DMSO.

3.4 *Hedgehog signalling is necessary, but not sufficient for RA-induced F9 cell differentiation*

Active Hedgehog signalling in F9 cells is required for RA-induced activation of both the canonical Wnt and Hedgehog pathways (Deol, Cuthbert et al.; *submitted*). This work shows that inhibition of the Hh pathway via cyclopamine, a chemical inhibitor of Smoothened, attenuated transcriptional activity of both the Wnt and Hh pathways using a luciferase assay for target genes of each pathway, even in the presence of RA. Activating the pathway via SAG, a chemical activator at the level of Smoothened, was not sufficient on its own to activate either pathway.

To determine whether this regulation of these pathways at the transcriptional level might translate to the protein level, cells were treated with either RA alone, SAG alone, or Cyclopamine and RA together. Results showed that RA and Cyclopamine caused a reduction in TROMA-1 (cytokeratin 8/18) and DAB-2 levels (1.5- and 5.15-fold change, respectively) relative to β -actin and compared to RA alone (1.71-, and 46.34-fold, respectively) (Fig. 4A and B). This reduction would indicate that cells had not differentiated since active Hh signalling in F9 cells was previously shown to be required to induce DAB-2 [9,46,47]. Unexpectedly, cells treated with SAG in the absence of RA did not show any significant increase in either TROMA-1 or DAB-2 (Fig. 4C and D). This latter result strongly suggests that activation of the Hh pathway alone is not sufficient to activate Wnt signalling or differentiate F9 cells.

The western blot analyses were corroborated by confocal immunofluorescence imaging, which showed TROMA-1 positive cells were only present in RA-treatments (Fig. 4E). Thus, while Hh signalling is required to induce F9 cells, it is not sufficient on its own for these cells to differentiate into PrE.

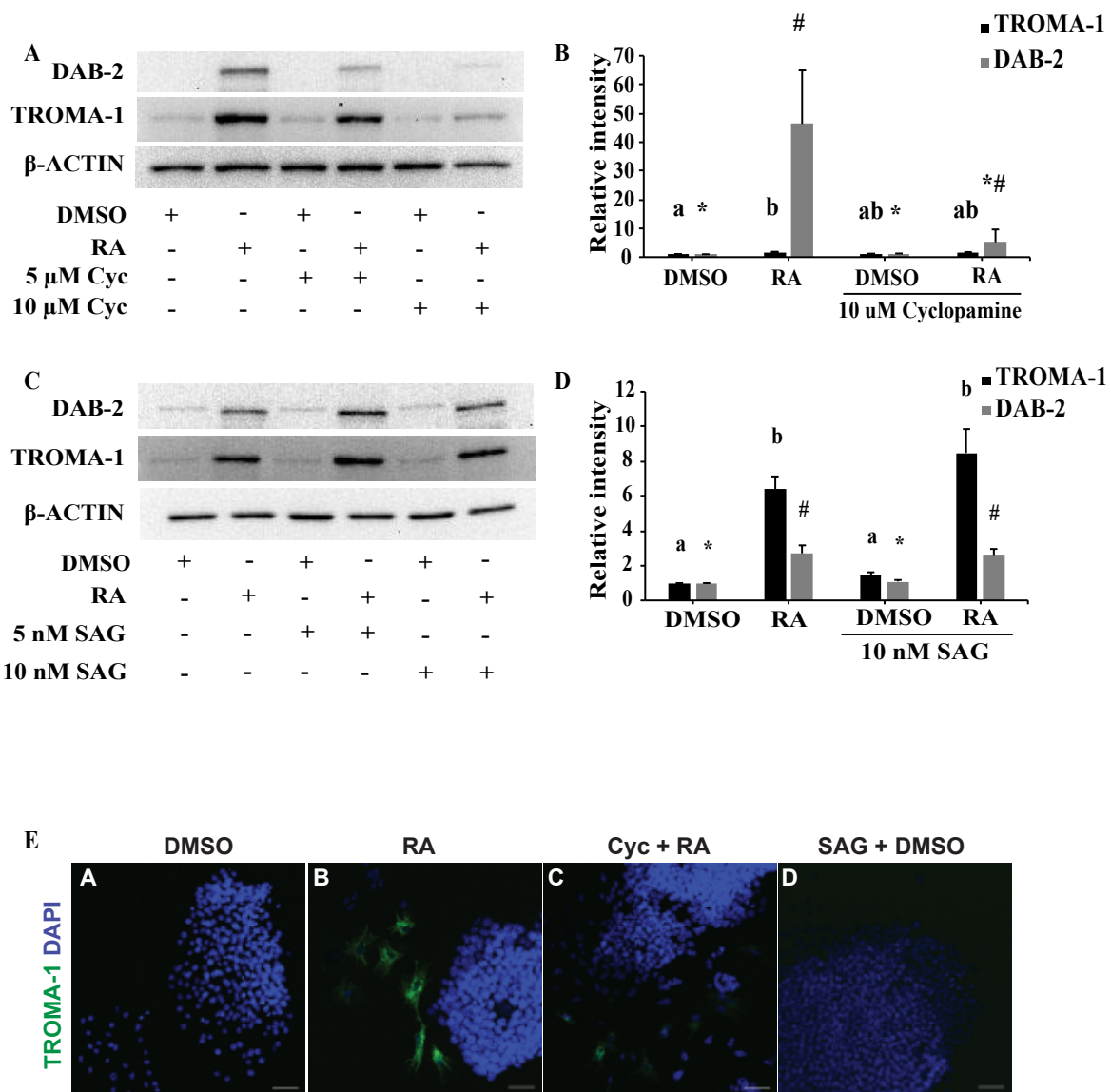
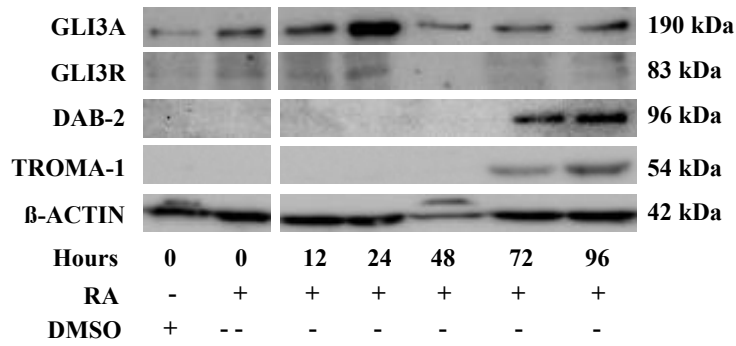


Figure 7. **Hedgehog signalling is necessary but not sufficient for RA-induced F9 cell differentiation.** F9 cells were treated with DMSO (negative control), RA (Positive control), (A) Cyc and RA, and (B) TROMA-1 and DAB-2 band intensity was quantified, or (C) SAG and DMSO, and again (D) TROMA-1 and DAB-2 band intensity was quantified. The data presented are from three independent experiments \pm SEM. Letters and #/* indicate significant difference ($p < 0.05$) as tested by One-Way ANOVA followed by a Tukey test relative to β -ACTIN and DMSO. (E) Cells were fixed at 96hr post-treatment and processed for immunocytochemistry using the TROMA-1 antibody to detect keratin-8 and counterstained with DAPI to detect nuclei. Scale bar is equal to 50uM.

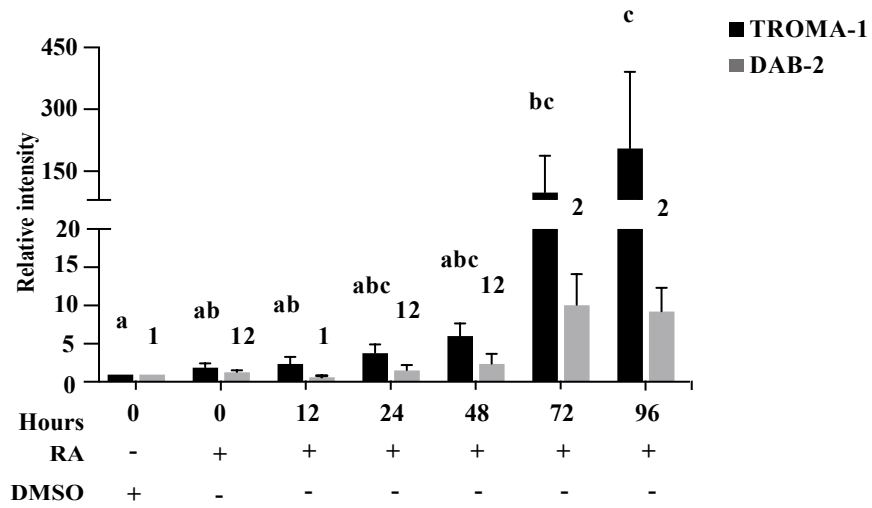
3.5 *GLI3A:GLI3R ratio changes during RA-induced F9 cell differentiation*

That Hh signalling was required, but not sufficient, to induce PrE points to a mechanism within the Hh pathway that controls differentiation, specifically one involving GLI3. GLI3 is a downstream transcription factor of the Hh pathway, which exists in either an active form (GLI3A; 190kDa) during active Hh signalling or in the predominantly repressor form (GLI3R; 83kDa) in the absence of signalling. Protein lysates were collected from RA-treated cells at 0, 2, 24, 48, 72, and 96 hours. As expected, signals for both TROMA-1 and DAB-2 were evident at 72 hours (Fig. 5A and B), as these are markers of positive PrE differentiation, while GLI3A and GLI3R were present in DMSO and RA treated cells from time 0. Interestingly, GLI3A levels as compared to DMSO appeared to increase, albeit not statistically significantly, between 24 and 48 hours post-RA ($p=0.084$), whereas there was a subsequent decrease in GLI3R levels, albeit non-significant, ($p=0.087$) (Fig. 5A and C). By 96 hours, however, both GLI3A and GLI3R levels were not significantly different from those seen in the DMSO and RA cells at time 0. These results indicate that a post-translational change in GLI3 occurred with RA treatment, and this is expected to have a positive impact on PrE differentiation, as noted by positive TROMA-1 and DAB-2 signals.

A



B



C

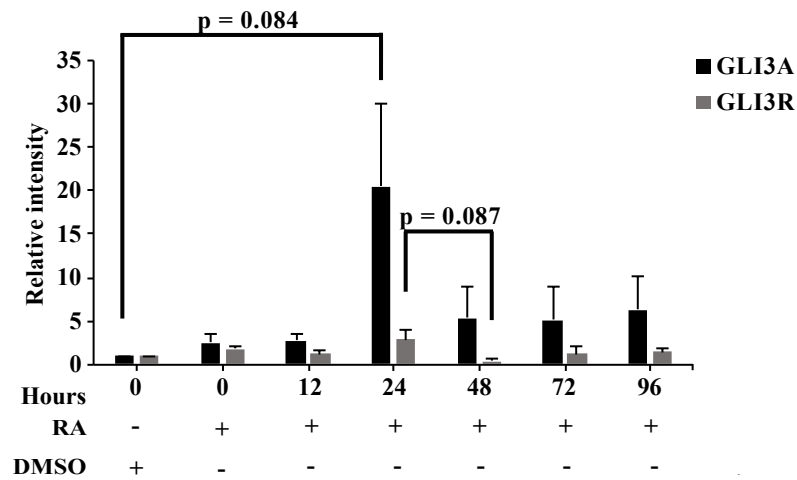
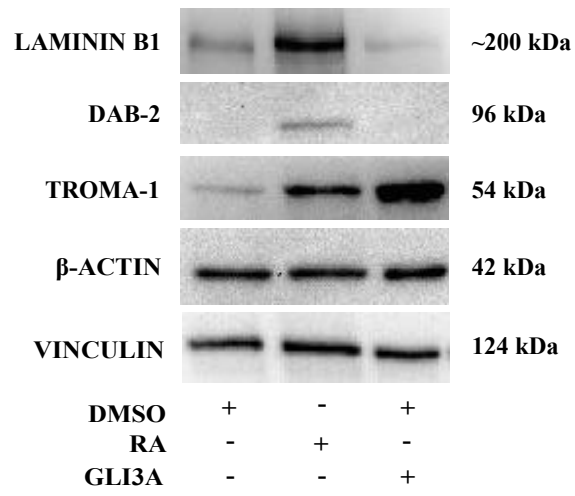


Figure 8. **GLI3A:GLI3R ratio changes during RA-induced F9 cell differentiation.** F9 cells were treated with DMSO and collected at 0 hour or 96 hour time points (negative control) or RA and collected at 0, 12, 24, 48, 72, and 96 hour time points and (A) probed for TROMA-1, DAB-2, GLI3A, and GLI3R and subsequently band intensity relative to β -ACTIN was quantified for (B) TROMA-1 and DAB-2 and (C) GLI3A and GLI3R. Letters and #/* indicate significant difference ($p < 0.05$), or appropriately labeled significance value, as tested by One-Way ANOVA followed by a Tukey test relative to β -ACTIN and DMSO.

3.6 *GLI3A overexpression is not sufficient to differentiate F9 cells to PrE*

The active or repressive state of the GLI transcription factors is dictated by the Hh pathway. Active Hh signalling at the level of Smo was not sufficient to differentiate cells, but because previous results (Fig. 7) indicated a RA-responsive role of GLI3 during PrE differentiation prior to the appearance of DAB-2 at 72 hours, it was hypothesized that overexpression of GLI3A would be sufficient to differentiate F9 cells to PrE. Cells were co-transfected with pcDNA3.1 empty vector and pcDNA3.1-*DN2Gli3* (GLI3A) constructs, with or without RA treatment, and assayed for differentiation using antibodies against TROMA-1, DAB-2, and LAMININ B-1 (a secreted extracellular matrix component and marker of general ExE). Interestingly, while overexpression of GLI3A in the absence of RA resulted in an increase in TROMA-1 (8.19-fold as compared to 3.61-fold with RA alone), neither DAB-2 or LAMININ B-1 were present in this treatment, as compared to RA-treatment alone (Fig. 6A and B). This result indicates that while GLI3A is capable of increasing the intermediate filament found in PrE and general ExE, it is not sufficient to fully differentiate these cells to the PrE lineage.

A



B

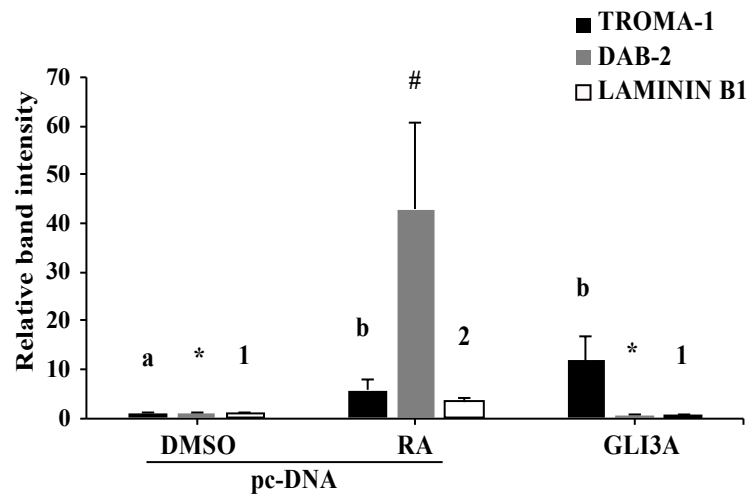


Figure 9. **GLI3A overexpression is not sufficient to differentiate F9 cells to PrE.** F9 cells were transfected with a pcDNA3.1 empty vector (control) or pcDNA3.1-*DN2Gli3* (GLI3A). Total protein was collected 96 hours later and (A) analyzed using immunoblot probing for TROMA-1, DAB-2, and LAMININ B1. (B) TROMA-1 and DAB-2 bands were quantified relative to β -ACTIN while LAMININ B1 bands were quantified relative to VINCULIN loading control. Letters and #/* indicate significant difference ($p < 0.05$) as tested by One-Way ANOVA followed by a Tukey test relative to β -ACTIN or VINCULIN and DMSO.

3.7 *GSK3- β inhibition is not sufficient for F9 cell differentiation*

Having previously identified a link between Hh and Wnt/ β -catenin in our lab, one possible candidate serving as a node to inhibit both pathways is GSK3- β [48,49]. Our lab previously demonstrated that both SAG and BIO treatment separately (the latter being an inhibitor of GSK3- β) caused a significant increase in *Gli* transcription activity, which was not significantly different from the RA controls (Deol, Cuthbert, et. al.; *submitted*). While 10 nM SAG treatment did not increase the activity of the Wnt pathway, 3 nM BIO alone was sufficient to increase TCF/LEF activity, as expected. Thus, inhibiting GSK3- β impacts positively on both pathways. These results demonstrate the sufficiency of GSK3- β inhibition to positively regulate both the Hh and Wnt pathways in F9 cells, but did not provide an indication as to whether this common activation by GSK3- β inhibition was sufficient for differentiation. To that end, it was of interest to treat cells with BIO and assay for differentiation using the TROMA-1 and DAB-2 markers. Protein lysates collected at 96 hours did not show any significant increase in either TROMA-1 or DAB-2, thereby indicating that GSK3- β inhibition, while sufficient for activation of both Hh and Wnt pathways, was not sufficient alone to induce F9 cells to form PrE.

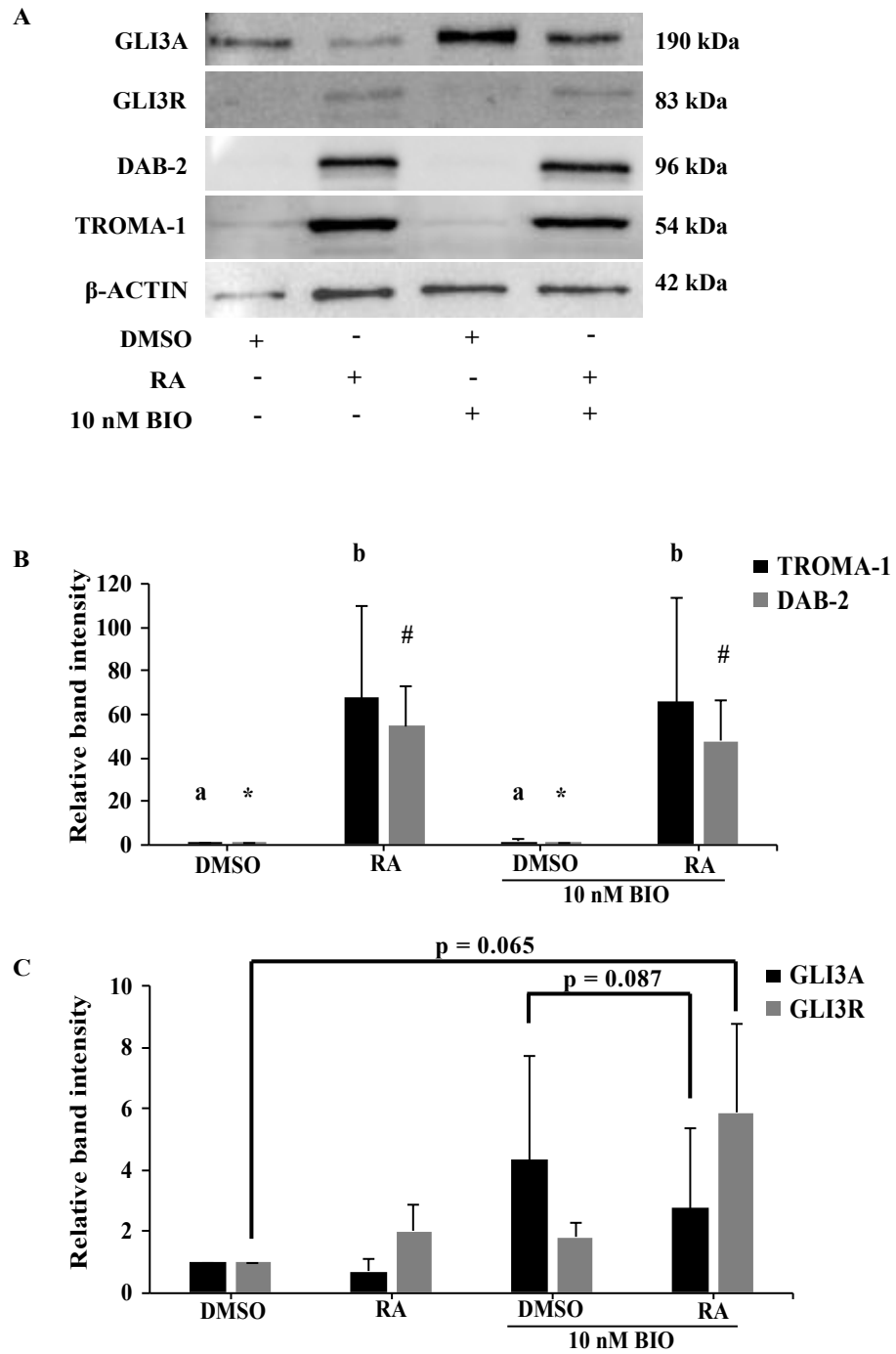


Figure 10. **GSK3- β inhibition is not sufficient for F9 cell differentiation.** F9 cells were treated with DMSO, RA, or DMSO and 5nM or 10nM of BIO, and protein lysate was collected at 96 hours post-treatment. (A) Immunoblot was probed for TROMA-1, DAB-2, GLI3A, and GLI3R. (B) TROMA-1 and DAB-2, as well as (C) GLI3A and GLI3R band intensity was quantified, relative to β -ACTIN. Letters and #/* indicate significant difference ($p < 0.05$), or appropriately labeled significance value, as tested by One-Way ANOVA followed by a Tukey test relative to β -ACTIN (loading control) and DMSO.

3.8 *Gai1* is expressed in E3.5 mouse blastocyst, and expression is altered during RA induced F9 cell differentiation

Several levels of post-translational phospho-regulation lie between Smoothed activation and GLI3 regulation in the Hh pathway, and it is generally understood that PKA acts to “prime” the GLIs for further regulatory modification by GSK3- β and CK1 [24]. Since MAPK and PKA inhibition is required for F9 cell differentiation to PrE [6], together with Hh and Wnt signalling [7,47], I next investigated whether inhibitory G proteins may play a role in this differentiation event. It is interesting to note that adenylyl cyclase activity is decreased in PrE as compared to undifferentiated and PE-differentiated F9 cells [35], indicating that PKA activity at this time would be expected to be low given the depressed levels of cAMP.

Preliminary work was conducted to determine whether *Gnai1* and *Gnai2* mRNA encoding the inhibitory G proteins G α i1 or G α i2, respectively, are expressed in F9 cells, and further to confirm expression of these genes in the mouse E3.5 blastocyst [50]. While *Gnai* expression has been previously investigated by Galvin-Parton et al. [35], these results contradict work by Williams et al. [50] regarding G α i protein and mRNA expression, citing lack of expression of *Gnai1* in the F9 cell model. For this reason, it was of interest to re-visit these results, investigating the expression of *Gnai1* and *Gnai2*. It was hypothesized that if an inhibitory G protein is responsible for the decrease observed in cAMP levels in PrE-differentiating F9 cells, a change in mRNA levels may be detected early in differentiation. If so, this would accommodate the necessary MAPK signalling via a decrease in PKA as well as modifications to GLI that occur by 24 and 46 hours (Fig 5A and C). mRNA from RA-treated F9 cells was collected and assayed for *Gnai1* and *Gnai2* at 24, 48, and 72-hours post treatment. Clearly both *Gnai1* and *Gnai2* genes are expressed in F9 cells (Ct values ranging from 22-25 and 20-23, respectively), however, only *Gnai1* increased significantly from 48-72 hours post-RA treatment (Fig. 8A). These results contradict results by Malbon et al. (1990) and agree with blastocyst expression results by Williams et al. (1996) in that both *Gnai1* and *Gnai2* were detectable in the E3.5 mouse blastocyst (Fig. 8B), once again confirming the presence of these mRNAs at the blastocyst stage. This work is preliminary, as G protein signalling is activity-dependent rather

than expression dependent, but confirms blastocyst expression results and establishes a potential future line of investigation for crosstalk in PrE differentiation.

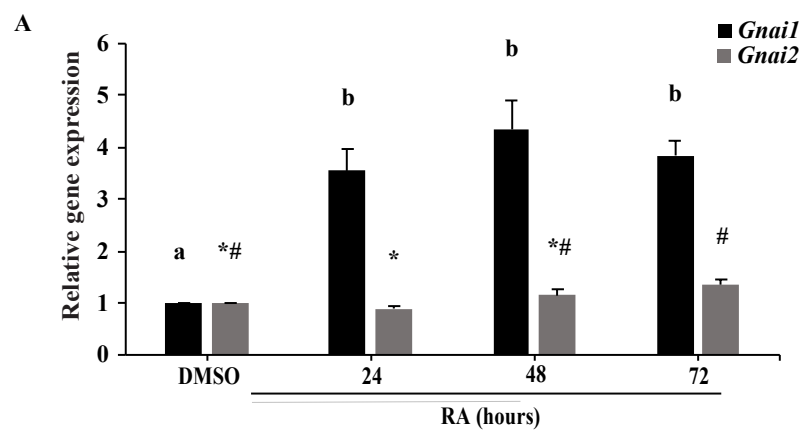


Figure 11. *Gai1* is expressed in E3.5 mouse blastocyst, and expression is altered during RA-induced F9 cell differentiation. Presence of *Gnai1* and *Gnai2* mRNA in PrE-differentiating F9 cells was assayed through collection of mRNA from (A) DMSO or RA-treated cells at 24, 48, and 72 hours post-treatment, as well as (B) cDNA from pooled E3.5 mouse blastocysts. Data are representative of three independent experiment, and each cDNA and protein lysate sample consisted of 25-35 pooled embryos.

CHAPTER 4

4 Discussion

Cellular signalling pathways play major roles in regulating developmental processes and one of these pathways critical in the formation of embryo involves the morphogen Hedgehog. Although many studies have investigated mechanisms controlling PrE differentiation, little is known of the involvement of Hh in ExE differentiation. By utilizing the F9 teratocarcinoma cell line, which models ExE differentiation to PrE via treatment with RA, this study was able to focus on defining and highlighting IHH control of these events [21]. That no detectable changes occurred in the expression of the other two *Hh* genes encoding the Sonic and Desert isoforms, would indicate that *Ihh* is the candidate gene up-regulated during differentiation in response to RA (Fig. 1), and this corroborates an earlier study with F9 cells [21]. I also assessed changes in the expression of some of the key components of the Hh pathway as many are Hh target genes up-regulated in response to active signalling [51]. Activation of Hh target genes occurs primarily as a result of the GLI1/GLI2 activator and/or a reduction of the GLI3 repressor. In this study significant changes were detected in *Gli1* and *Gli3* expression in response to RA, the latter a known Hh target gene [52]. While statistically significant changes (either increasing or decreasing) were observed for both *Gli1* and *Gli3*, these changes were less than 3-fold and therefore may not be biologically significant, particularly as the protein product of these genes is post-translationally regulated. The increase in *Ptch2* expression following RA treatment (Fig. 1) was expected, as this gene is commonly used as an indicator of Hh signalling [53] and plays a clear role in chick early embryonic development, over that of *Ptch1* [39]. That the expression of some of the Hh components were not affected by RA, such as the Gli transcription factors, might suggest that their regulation during the differentiation of PrE is at the protein level. This supposition is currently being tested, but it may be difficult to come to clear conclusions given that some of these components are proteins whose activity are much more diagnostic than proteins levels themselves.

Once it was established that *Ihh* is expressed in the F9 cell model through my studies as well as previously by the Grabel Lab [17,21], it was very much of interest to determine that this

gene and its protein product are in fact present in the appropriate stage of mouse blastocyst development, ie E3.5. My results clearly demonstrate that *Ihh* mRNA is in fact expressed in the E3.5 mouse blastocyst, and sequencing of the amplicons confirmed the identity of *Ihh* (Fig. 2A). Secondly, it was established that the IHH protein is detectable in the E3.5 mouse blastocyst (Fig. 2B), supporting my hypothesis that this ligand plays a role in the differentiation of PrE through RA treatment in F9 cells and during mouse blastocyst development from E3.5.

RA treatment of F9 cells regulates a plethora of genes [7,53,54] including *Gata6*, which as described plays an integral role in patterning the extraembryonic and embryonic endoderm [1,55]. In F9 cells *Gata6* expression increases directly in response to RA, and when translated it binds to the *Wnt6* promoter [9]. *In silico* analysis revealed the *Ihh* promoter contains putative GATA6 binding sites [Appendix 3; 22], suggesting that the gene is also regulated directly or indirectly by this transcription factor. Previous experiments were conducted by our lab to test if *Gata6* expression would activate the Hh pathway. Results showed that *Gata6* overexpression led to a significant increase in *Gli* reporter activity relative to the empty vector (Deol, Cuthbert et al.; *submitted*), placing GATA6 in a signalling hierarchy upstream of *Wnt6* [3] and Hh signalling in PrE formation. Thus, since *Ihh* is up-regulated in response to RA (Fig. 1), and that RA up-regulates *Gata6* [3], which in turn activated the Hh pathway (Deol, Cuthbert et al.; *submitted*), the existence of a putative GATA6 binding site in the *Ihh* promoter prompted us to test for a possible link between these players during the differentiation of PrE. F9 cells were transfected with a *Gata6* expression vector and qRT-PCR analysis showed the 250-fold increase in *Gata6* expression (Fig. 3A) and caused a concomitant increase in the expression of *Wnt6* and *Dab2* (Fig. 3B and C), which we have shown previously to accompany PrE formation [10,45,56]. Overexpression of *Gata6* was also sufficient to increase *Ihh* relative to the DMSO control (Fig. 3D). While this study is the first to show the relationship between *Gata6* and *Ihh* signalling in this early developmental event it should be noted that others have reported the converse in other systems, where GATA6 represses *Shh* expression [57,58]. Nevertheless, having established that a hierarchy exists involving RA, *Gata6*, *Wnt6* and *Ihh*, the crosstalk between Wnt and Hh was examined further to determine if inhibiting or activating the Hh pathway would affect PrE formation.

The smoothened agonist (SAG) and cyclopamine (Cyc) are a well-characterized activator and inhibitor, respectively, of the Hh pathway, and initial experiments previously completed in our lab demonstrate that both chemicals were active, and affect the *Gli* reporter in F9 cells (Deol, Cuthbert et al.; *submitted*). These results were encouraging and suggested that the Hh pathway was sufficient and/or necessary for PrE formation. F9 embryonal carcinoma cells respond to RA by increasing the levels of DAB-2 [56,59], and cytokeratin 8/18 (TROMA-1) [60], and this was evident by immunoblot analysis (Fig. 4A and 4C). Cyclopamine had a dramatic effect on reducing the levels of these markers induced by RA, and also suggested that active Hh signalling must promote PrE differentiation (Fig. 4A and B). Although very few studies have investigated Cyc and *Dab2* in this context, there is evidence for activation of the Hh pathway with purmorphamine, another Smo agonist, showing reduced expression of the *Dab2* marker in developing zebrafish venous cells [61]. Since previous work by our lab demonstrated the ability of Cyclopamine treatment to attenuate both *Gli* and *TCF-LEF* activity as well as the Wnt-target protein DAB-2 (Deol, Cuthbert et al.; *submitted*), crosstalk between these pathways, contributing to PrE differentiation, is suggested. This crosstalk may be between common downstream signalling targets or between common regulatory nodes between these pathways, such as GSK3- β .

SAG treatment of F9 cells had no apparent positive or negative effect either alone or in combination with RA on DAB-2 protein level (Fig. 4C and 4D), which would indicate that it is not a direct target of Hh. The same appears to be true for cytokeratin 8/18 as detected with the TROMA-1 antibody (Fig. 4E), which again given the paucity in the literature, contradictory results have shown increases in cytokeratin 8/18 expression due to Shh signalling in mouse and human cells [62,63]. Thus, despite the fact that PrE markers were absent when the Hh pathway was blocked in F9 cells, it is difficult to provide a compelling explanation for why these markers did not appear in the presence of active Hh signalling. One possibility, however, is that the major contribution by Hh signalling is not for the activation of genes required to induce markers of PrE differentiation, but rather the activation or inhibition of some other component(s) that act with the Wnt target genes required to induce PrE. To investigate this further, previous experiments were completed by our lab, transfecting F9 cells with a Wnt/ β -catenin dependent pBARL reporter, a luciferase construct responsive to the Wnt pathway target TCF/LEF, to assess

downstream Wnt transcriptional activation and its activity measured in response to active (SAG) or inactive (Cyc) signalling (Deol, Cuthbert et al.; *submitted*). While RA and BIO were successful—as positive controls; the latter serving to inhibit GSK3- β activity—SAG was unable to activate the pBARL reporter. Nevertheless, the attenuation of RA-induced pBARL activity by Cyc pointed initially to active Hh signalling being required to cooperate positively with the Wnt/ β -catenin pathway that we and others have shown to be required for F9 cells to form PrE [10,34]. Since a variety of compounds act positively or negatively on both pathways [64–66], an alternative explanation is that Cyc may be acting in an inhibitive manner on both Wnt and Hh signalling. In F9 cells the inability of SAG to induce Wnt target genes (Fig. 4C and D) or to influence the pBARL promoter (Deol, Cuthbert et al.; *submitted*) would indicate that Hh is not signalling directly upstream of Wnt, and the mechanism(s) by which Cyc blocks cytokeratin 8/18 and the Wnt target DAB-2 remains to be determined.

Since Hh signalling was determined to be required but not sufficient to differentiate F9 cells to PrE, and because inhibition of this pathway resulted in attenuation of DAB-2 of the Wnt pathway, it is suggested that this crosstalk occurs in parallel. As such, it was hypothesized that Hh activation by RA treatment may be required for relief of inhibition on the Wnt pathway, as much as transcriptional activation of its own targets. As GLI3 transcription factor is generally thought to exist in a repressor form in the absence of Hh and active form in the presence of Hh, this specific GLI protein was interrogated. F9 cells were treated with RA and analyzed at time points between 0 and 96 hours. While TROMA-1 and DAB-2 showed expected results, increasing at 72 hours, it was also observed that GLI3A does increase with RA treatment by 24 hours, and GLI3R has a temporary decrease between 24 and 48 hours (Fig. 5). These results are important, as GLI proteins, once post-translationally modified via phosphorylation and/or partial proteosomal degradation, are relatively unstable [24,25]. In demonstrating an alteration in GLI protein state, it is established that not only is Hh signalling required for activation of the Wnt pathway, but that the Hh pathway is likely required for its own traditional signalling as well. It is important to note, that while GLI3 protein does act as a transcription factor, it also interacts physically with both Nanog [26] and β -catenin, whereby Hh signalling must be active to allow GLI3R to release β -catenin to move to the nucleus, even in the presence of Wnt [27]. While unclear whether these interactions do occur in the F9 cell model, preliminary results are

encouraging, demonstrating a requirement for active Hh and therefore a decrease in the repressive GLI protein.

GLI3A, having been shown to increase with RA treatment by 24 hours, continued to be a potential target of investigation. While Hh signalling alone via SAG activation was not sufficient for PrE differentiation, several regulatory steps lie between activation of Smoothed and the final activator-to-repressor state of GLI in the cell including PKA, GSK3- β , and CK1 [14]. We therefore concluded that by-passing this GLI3A level of the pathway might be sufficient to differentiate F9 cells to PrE. Unfortunately, this did not prove to be the case, as overexpression of GLI3A did increase TROMA-1 well over that seen by RA alone, but neither PrE markers DAB-2 or LAMININ B1 signal were observed (Fig. 6).

Despite not having convincing evidence to explain Cyc and SAG results, the data does point to the existence of crosstalk between Wnt/ β -catenin and Hh signalling. Previous work by our lab examined this potential crosstalk in more detail, specifically targeting a node in regard to PrE formation. F9 cells were transfected with a *Gli* reporter and treated with BIO (Deol, Cuthbert et al.; *submitted*) and an increase in the activity of this reporter and the *TCF/LEF* reporter with BIO suggest GSK3- β as this node. To follow this work, it was hypothesized that targeting GSK3- β as a possible node of common regulation might result in F9 cell PrE differentiation. This again proved not to be the case, resulting in an absence of TROMA-1 or DAB-2 PrE marker signal (Fig. 7A and B). Further, inhibition of GSK3- β appeared to result in a decrease in GLI3A in cells co-treated with RA as compared to DMSO, as well as an increase in GLI3R (Fig 7A and C). These results would indicate that inhibition of GSK3- β acts to suppress Hh signalling, rather than activate during F9 cell PrE differentiation, in contradiction with previous results showing increased *Gli* luciferase activity with BIO treatment (Deol, Cuthbert et al.; *submitted*). A possible explanation for these results exists in simplicity: time. While BIO treatment does modestly decreases GLI3A levels in the presence of RA, it is important to note that these results reflect the 96hr time-point post-treatment. Previous results demonstrate an important window for GLI regulation between 24 and 48 hours, suggesting that decreased GLI3A seen in Fig. 7 may reflect “rebound” signalling. Such explanation does have precedence, as NANOG and GLI3 physically interact in the mouse EC stem cell, whereby each transcription

factor acts as a “buffer” balancing the cell between a pluripotent and differentiating state until intervening signalling tips the balance [26]. There is further precedence for this suggestion, as BIO can activate *Gli1* transcription in human breast cancer cells [62], and GSK3- β silences Hh signalling by phosphorylating the *Drosophila* Gli homologue, Cubitus interruptus (Ci) following a previous priming phosphorylation by PKA [48]. When Hh is absent in *Drosophila*, GSK3, PKA and casein kinase 1 alter the activity of Ci by interacting with intermediates that enhance the proteolytic degradation of Ci into its transcriptionally repressive form [67]. This overall regulation of Ci/Gli and Wg/Wnt by GSK3 may be highly conserved [68], and it appears this conservation could also exist in F9 cells.

Several levels of phospho-regulation lie between Smoothed activation and GLI3 regulation in the Hh pathway, and it is generally understood that PKA acts to “prime” the protein for further regulatory modification by GSK3- β and CK1 [24]. In addition to Wnt and Hh signalling, it is established that the MAPK pathway is required for F9 PrE differentiation via inactive PKA signalling [6]. Given that F9 cells demonstrate a decrease in cAMP levels (downstream of PKA—during PrE differentiation as compared to undifferentiated) and the subsequent PE differentiation, it was plausible that PKA may be actively regulated during this differentiation state. Not only would this regulation allow for MAPK signalling, but would also account for altered GLI state in the Hh pathway. Preliminary work was done to determine the basic validity of this hypothesis, determining the expression of both *Gnai1* and *Gnai2* in both undifferentiated and PrE differentiated F9 cells (Ct values ranging from 20-25; Fig. 8A) as well as the E3.5 mouse blastocyst (Fig. 8B). Furthermore, the expression results during differentiation of F9 cells deviate from that by [35,69] that claim *Gnai1* is not expressed in the F9 cell line or mouse blastocyst. It is established that in the mammalian cell the 12-pass transmembrane protein SMO, responsible for regulation of the Hh pathway, is capable of interacting with G proteins as a GPCR [38]. While not extending to the protein level, the preliminary results presented here support the possibility that inhibitory G proteins are expressed at the appropriate time point to play a critical role in the crosstalk involved in PrE differentiation.

Together, the data supports the view that the differentiation of F9 cells to PrE requires the cooperative activity of at least two, and potentially three signalling pathways. Specifically, the

activities and subsequent regulation of Hh and Wnt target genes required for this differentiation may be facilitated by phosphorylation modulation by GSK3- β and/or G protein regulation. Although we have identified one node that is shared between these two pathways, given the complex interplay between them as evident from the Cyc results, it is likely that others exist.

I propose a model for this PrE differentiation signalling whereby RA results in the increase of *Gata6* and subsequently Wnt and Hh ligands WNT6 and IHH. I propose that active Hh signalling elicits a change from an overall GLI3R to GLI3A state, resulting in activation of target genes as well of relief of inhibition on the Wnt pathway through yet-to-be-determined means. Finally, I propose a node of common control between these pathways at the level of GSK3- β and/or G protein signalling, resulting in differentiation of PrE (Fig 9). As further investigation is required to fill in the blanks of this proposed model, our lab is currently continuing this work to evaluate other candidates that may provide the additional crosstalk that fine tunes the regulation of the Wnt and Hh pathways during extraembryonic endoderm development.

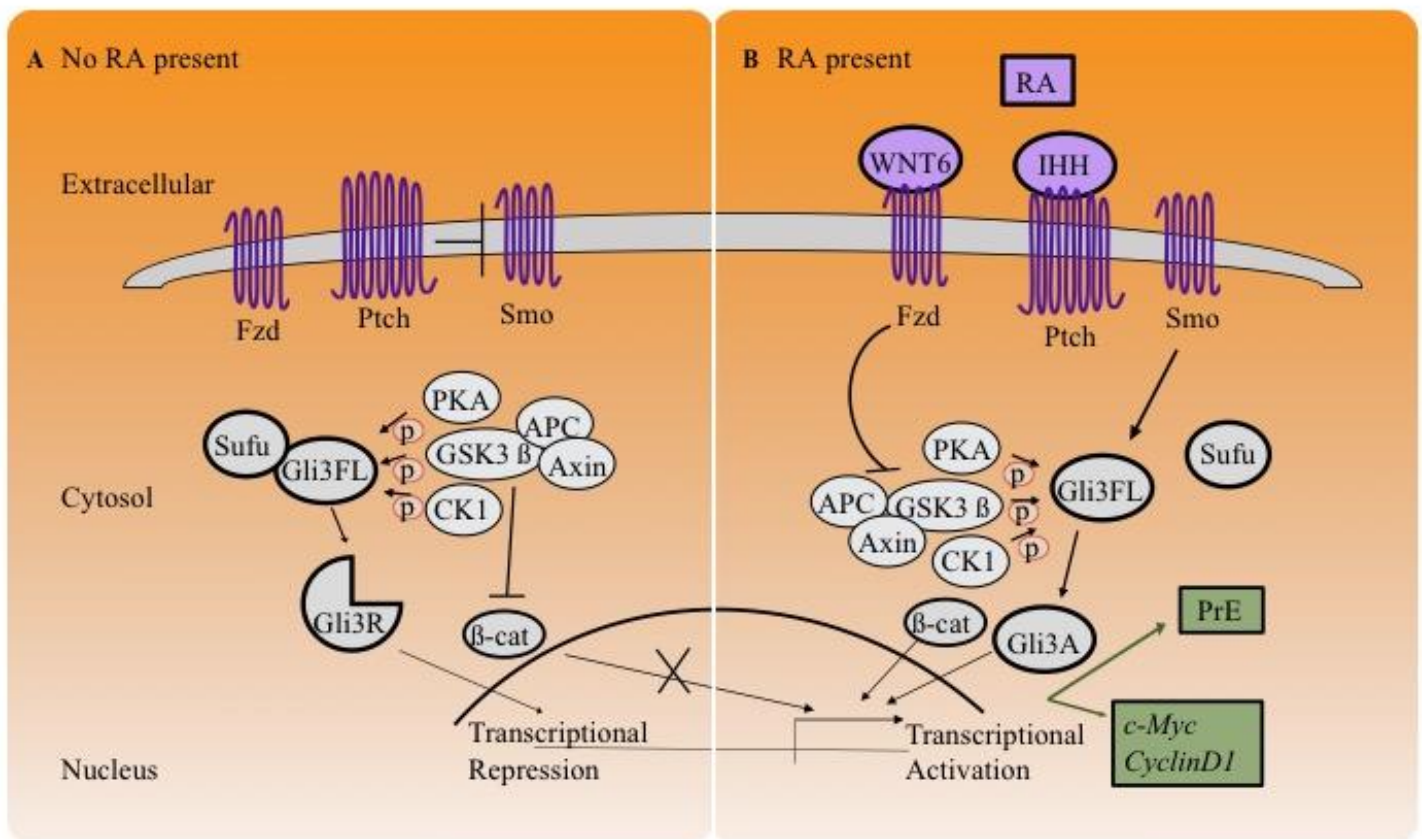


Figure 12. A model for primitive endoderm differentiation in F9 cells. In the (A) absence of RA, canonical/ β -catenin Wnt signalling is in an off-state resulting in phosphorylation of β -catenin by the destruction complex (GSK3- β , APC, and Axin) and its proteosomal degradation. Similarly, in the absence of RA, Hedgehog signalling is inactive, resulting in phosphorylation of GLI3FL and subsequent formation of its repressive transcription factor (GLI3R) moving into the nucleus. (B) In the presence of RA, canonical/ β -catenin Wnt signalling is in the on-state, resulting in inhibition of the destruction complex, allowing β -catenin to move to the nucleus to act as a transcriptional activator, contributing to PrE differentiation. Similarly, the Hedgehog signalling pathway is also in the on-state, resulting in alternative phosphorylation of GLI3FL, forming an increase in the active transcription factor (GLI3A) by 24 hours post-treatment to activate target genes, contributing to differentiation.

Limitations and Future Directions

As in all investigation with a cellular model system, there are limitations to the scope of this study. As demonstrated in Fig. 7E, efficiency of differentiation of F9 cells treated with RA, while not quantified here, is visually observed to be lower than 100%. This means that not all cells treated with RA in experimental groups are homogeneously differentiated; a result that is likely reflected in immunoblot analysis. In particular, a limitation of this combined differentiation efficiency and use of immunoblot analysis is evident in Fig 8. as Gli3 antibody (R&D Systems, AF3690) is not specific to multiple epitopes of phosphorylation sites. This means that this antibody is unable to discriminate between neutral full-length and active form of this transcription factor, limiting the conclusion as to the activity of this protein under undifferentiated/differentiated states. Similarly, conclusions possible by mRNA analysis for inhibitory G proteins 1 and 2 in Fig. 11 is limited, as these genes code for enzymes. While mRNA changes were detected, the translation of this information to functional and active enzymes in the cell at these steady-state mRNA levels is unknown. As well, demonstration of a positive regulation of *Ihh* mRNA by GATA6 overexpression here does not conclude whether this regulation is direct, or indirect through other signalling molecules in the pathway.

Future directions for this work involves expansion of studies applied to each investigated pathway; Wnt, Hh, and MAPK individually as well as crosstalk between these pathways. Several future investigations are possible to address the limitations of this study. Mass Spectrometry may be used to discern between GLI3 protein modifications in varying differentiated cell states. Of value, would be the investigation of Gai protein levels in differentiated and undifferentiated cell

states through immunoblot, as well as the determination of the activity of these proteins through cAMP/PKA activity assay with pertussis toxin (inhibitory G protein inhibitor). Finally, it was undetermined in this study whether GATA6 regulation of *Ihh* is direct or indirect, and it would therefore be of value to complete a Chomatin-Immunoprecipitation experiment to answer this question.

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Appendices

Appendix 1. Forward and reverse primer sequences

Gene	Forward Primer	Reverse Primer	Source doi
Smoothened	CCTGACTTTCTGCGTTGC	GGTCTGACACTGAATCCC	10.3109/10520295.2011.602645
Patched1	TGCTGTGCTGTGGTCATCCTGATT	CAGAGCGAGCATAGCCCTGTGGTTC	10.1016/j.ydbio.2014.05.014
Patched2	CCCGTGGTAATCCTCGTGGCCTCTAT	TCCATCAGTCACAGGGGCAAAGGTC	10.1016/j.ydbio.2014.05.014
Gli1	GGAAGTCCTATTCACGCCTTGA	CAACCTTCTTGCTCACACATGTAAG	10.1186/1471-2121-9-49
Gli2	TACCTCAACCCTGTGGATGC	CTACCAGCGAGTTGGGAGAG	10.1242/dev.067264
Gli3	GCTCTTCAGCAAGTGGTTCC	CTGTGGGCTTAGGATCTGTTG	10.1038/labinvest.3700537
L14	GGGTGGCCTACATTTCCTTCG	GAGTACAGGGTCCATCCACTAAA	Harvard primer bank
Shh	TCCTGTGAAAGCAGAGAACTCC	GGGACGTAAGTCCTTCACCA	10.1371/journal.pone.0044121
Dhh	ACCCCGACATAATCTTCAAGGAT	GTACTCCGGGCCACATGTTC	10.1016/j.modgep.2004.05.001
Ihh	GACTCATTGCCTCCAGAAGT	CCAGGTAGTAGGGTCACATTGC	10.1002/jbmr.443
Gata6	ATGGCGTAGAAATGCTGAGG	TGAGGTGGTCGCTTGTGTAG	10.1128/NCB.25.7.2622-2631.2005
Wnt6	GCAAGACTGGGGTTCGAG	CCTGACAACCACACTGTAGGAG	Harvard primer bank
Dab2	GGAGCATGTAGACCATGATG	AAAGGATTTCGAAAGGGCT	doi:10.1038/sj.onc.1210829
Sufu	CGGACCCCTTGGAATGTTA	CTTCAGACGAAACGTCAACTCA	Harvard primer bank
CCnd1	GCGTACCCTGACACCAATCTC	ACTTGAAGTAAGATACGGAGGGC	doi:10.1038/ncomms3544
c-Myc	CGGACACACAACGTCTTGAA	AGGATGTAGGCGGTGGCTTTT	doi:10.1038/srep22966

Appendix 2. DMSO (96h) mRNA Ct values at 96 hours

Gene	Average DMSO Ct Value
<i>Ihh</i>	28.84
<i>Shh</i>	35.27
<i>Dhh</i>	33.57
<i>Wnt6</i>	33.15
<i>Gata6</i>	27.39
<i>Dab2</i>	28.70
<i>Ptch1</i>	24.28
<i>Ptch2</i>	27.74
<i>Gli1</i>	25.65
<i>Gli2</i>	24.39
<i>Gli3</i>	27.18
<i>Ccnd1</i>	25.93
<i>c-Myc</i>	24.69

Appendix 3. Putative GATA6 binding sites in *Ihh* promotor

Putative GATA6 Binding Site in the mouse *Ihh* promoter.*

chr1: 74996438-74996447

5' -TGATTGCCAGCCTGTGGCCCGGATCCTTATCTGCATTCTCGGCGCCAGCCCGGC-3'
 3' -ACTAACGGTCGGACACCGGGCTAGGAATAGACGTAAGGAGCCGCGGGTCGGGCCG-5'

* After Qiagen- SABiosciences.

Curriculum Vitae

Education

Master of Science 2015- 2017

Biology
Collaborative Graduate Program in Developmental Biology
University of Western Ontario, London, Ontario

Bachelor of Science 2011-2015

Honors Specialization in Biology
University of Western Ontario, London, Ontario

Pre-Health Science Certificate 2010

Georgian College, Barrie, Ontario

Research Experience

Graduate Research 2015-2017

Dr. Gregory M. Kelly Lab
University of Western Ontario, London, Ontario

Research Assistant 2015

Dr. Alexander Timoshenko Lab
University of Western Ontario, London, Ontario

Independent Study in Biology 2014-2015

Dr. Alexander Timoshenko Lab
University of Western Ontario, London, Ontario
Thesis title: Cytotoxic effect of galectin inhibitor thiodigalactoside on human HT-29
colorectal adenocarcinoma cells

Work Study 2012-2013

Dr. Sashko Damjanovski Lab
University of Western Ontario, London, Ontario

Research Work Contributed

Hedgehog signaling dynamics in extraembryonic endoderm differentiation. Deol, J, Cuthbert
TN, Hilton L, Kelly GM. (*Submitted*). Department of Biology, Western University.

Canadian National Perinatal Research Meeting **February 2017**
Montebello, Quebec

Hedgehog signaling is required during extraembryonic endoderm differentiation. Cuthbert TN, Spice DM, Kelly GM. Department of Biology, Western University.

Society of Developmental Biology Conference **August 2016**
Boston, Massachusetts, US

Hedgehog signaling dynamics in extraembryonic endoderm differentiation. Cuthbert TN, Kelly GM. Department of Biology, Western University.

Canadian Cancer Research Conference **November 2015**
Montreal, Quebec, Canada

Differential expression of galectins in cancer cells treated with stress stimuli mimicking tumor microenvironment. Vinnai J, Lewis J, Cuthbert TN, Timoshenko AV. Department of Biology, Western University.

Academic Honours and Awards

Graduate Student Teaching Award Nomination (2)	2017
Mitacs Research Grant	2017
Western Graduate Research Scholarship	2015, 2016
Dean's Honor List	2010, 2012, 2015
Hailon Award	2013-2014

Teaching Experience

First year Biology Laboratory (Biology 1001)	2015
Scientific Methods in Biology (Biology 2290)	2015
Advanced Developmental Biology (Biology 4338)	2016
Developmental Biology (Biology 3338)	2016
Advanced Cell Biology (Biology 3316)	2017

Training and Certificates

Basic Animal Care and Use Certification	2016
Laser Safety	2016
Comprehensive WHMIS Training	2016
Netiquette	2016
Open Educational Resources	2016
Designing Your Own Course	2016
General Laboratory Safety & Hazardous Waste Management Training	2014
Worker Health and Safety Awareness	2014
Biosafety Training	2014